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The analysis of nicotine-1′-*N*-oxide in urine, in the presence of nicotine and cotinine, and its application to the study of *in vivo* nicotine metabolism in man

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A rapid quantitative assay for nicotine-1′-*N*-oxide in urine, in the presence of nicotine and cotinine, is reported. The urinary excretion of nicotine, cotinine and nicotine-1′-*N*-oxide was determined after nicotine had been administered in cigarette smoke, orally, or intravenously to subjects with either fluctuating, controlled acidic or controlled alkaline urinary pH. The urinary excretion of the *N*-oxide in 24 h from smokers under normal conditions was about half that of the cotinine excretion; more *trans*- than *cis*-diastereoisomer of nicotine-1′-*N*-oxide was excreted.

Cotinine is the major basic urinary metabolite of nicotine in man (McKennis, 1965); recently Booth & Boyland (1970) demonstrated nicotine-1′-*N*-oxide in human urine. A quantitative method of analysis for nicotine-1′-*N*-oxide in urine, in the presence of nicotine and cotinine has been developed and used to examine the excretion of cotinine and nicotine-*N*-oxide in man administered nicotine by various routes.

MATERIALS AND METHODS

Materials

(—)-Nicotine (base and hydrogen (+)-tartrate salt) and titanium trichloride solution, technical grade 12·5% w/v $TiCl_3$ in *ca* 15% w/v HCl (total) were obtained from BDH Ltd. (—)-Nicotine-1′-*N*-oxide* was synthesized according to Taylor & Boyer (1959), (—)-nicotine-di-*N*-oxide and (—)-nicotine aryl mono-*N*-oxide according to Johnson, King & Turner (1958), and (—)-cotinine to Bowman & McKennis (1959).

Nicotine and cotinine were extracted and analysed as described by Beckett & Triggs (1966) using a Perkin Elmer F 11 gas liquid-chromatograph with a flame ionization detector.

Determination of the diastereoisomeric (—)-*nicotine*-1′-N-*oxides in urine in the presence of* (—)-*nicotine and* (—)-*cotinine*

Nicotine-1′-*N*-oxide* was reduced to nicotine with titanium trichloride at room temperature.

Replicate urine samples (5·0 ml) containing added nicotine-1′-*N*-oxide (10·0 μg/ml) were placed in centrifuge tubes and 5N hydrochloric acid (0·2 ml) and titanium trichloride solution (0·2 ml) added (Brooks & Sternglanz, 1959). At intervals between 5 min and 24 h after the addition of titanium trichloride solution the reduction of each sample was stopped by the addition of 5N sodium hydroxide (0·5 ml).

* 7:3 mixture *trans*- and *cis*-diastereoisomers (Beckett, Gorrod & Jenner, to be published).

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Replicate urine samples (5·0 ml) containing nicotine-1′-*N*-oxide (10·0 μg/ml) were reduced for 1 h as above and analysed for nicotine by g.l.c. (Beckett & Triggs, 1966). The peak height ratio obtained was compared with that for the theoretically equivalent amount of nicotine added to urine and assayed by the same procedure.

Using this procedure, a calibration curve for nicotine-1′-*N*-oxide between 0·1 and 10·0 μg/ml in urine was constructed. Again, the peak height ratios obtained were compared with those obtained for the theoretical equivalent of nicotine. Replicate urine samples (5·0 ml) containing either nicotine or cotinine (10 μg/ml) were similarly assayed but after a reduction time of 24 h.

*General procedure for the determination of the nicotine-*1′-N-*oxide, nicotine and cotinine content of urine*

Urine samples (4·0 ml) internal marker (1·0 ml; phendimetrazine 10 μg/ml) and 5N sodium hydroxide (0·2 ml) were placed in a centrifuge tube. Any nicotine present was extracted with diethyl ether (3 × 2·5 ml) and assayed by g.l.c. (Beckett & Triggs, 1966). The aqueous layer was washed with diethylether (2 × 2·5 ml) and then 5N hydrochloric acid (0·2 ml) added followed by titanium trichloride solution (0·2 ml). After 1 h, 5N sodium hydroxide (0·5 ml) and the internal marker (1·0 ml) were added and the solution assayed for nicotine as above. Cotinine was then extracted with dichloromethane (3 × 2·5 ml) after the addition of internal standard (1·0 ml; lignocaine 10 μg/ml) and assayed by g.l.c. (Beckett & Triggs, 1966). Blank samples of urine and standard solutions of nicotine-1′-*N*-oxide in urine (10·0 μg/ml) were assayed immediately by the above procedure and at intervals during two weeks.

*Detection of nicotine-*1′-N-*oxide in the urine of a smoker*

A 24 h urine collection from a heavy smoker (40 cigarettes/day) was concentrated to a small volume under vacuum at 70° using a rotary film evaporator and the residue freeze dried. The remaining viscous liquid was streaked across the origin of sheets of Whatmans 3MM paper and run in descending system overnight using n-butanol–n-propanol–2N ammonia (2:1:1 v/v) as solvent to separate the *cis*- and *trans*-diastereoisomers of nicotine-1′-*N*-oxide (Booth & Boyland, 1970). Reference samples of synthetic nicotine, cotinine, nicotine-1′-*N*-oxide, nicotine-di-*N*-oxide and nicotine aryl mono-*N*-oxide were also run. After elution, the papers were examined under ultraviolet light (Hanovia CHI 291) and compounds located by either Dragendorff or Iodoplatinate spray reagent (Smith, 1960). Papers were cut into strips according to the fluorescent bands present. These were shredded and then eluted with methanol containing 3% v/v ammonia (S.G. 0·880). The eluates were evaporated to dryness under vacuum at 40° using a rotary film evaporator. The residue was dissolved in distilled water (4·0 ml) and assayed for *N*-oxide metabolites.

Trials

The subjects were healthy males between 20 and 40 years. Smokers ceased smoking at least 36 h before the trial and the analysis of a "blank" sample of urine was used to demonstrate the absence of nicotine, nicotine-1′-*N*-oxide and cotinine.

Control of urinary pH. Urinary pH was maintained acidic (pH 4·8 ± 0·2) by prior administration of enteric coated ammonium chloride tablets (Beckett & Tucker, 1966). An alkaline urinary pH was maintained by an equivalent dosage regimen of sodium bicarbonate in aqueous solution.

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Table 1. *R_F values of (*
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Compound	A
Nicotine di-*N*-oxide ..	
cis-Nicotine-1′-*N*-oxide	
trans-Nicotine-1′-*N*-oxide	
Nicotine aryl mono-*N*-oxide	
Cotinine	
Nicotine	

* *cis*-Nicotine-1′-*N*-oxide d

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Urine collection. Urine samples were collected at 30 min intervals for 4 h after drug administration, at 60 min intervals for a further 8 h, then at will until a final sample was collected at 24 h, unless otherwise stated. The pH and volume of each sample was measured immediately and all samples were stored at 4° until analysed.

Oral trials. (—)-Nicotine hydrogen (+)-tartrate in aqueous solution (6·14 mg ≡ 2·0 mg base) was swallowed by subjects with acidic, alkaline or fluctuating urinary pH.

Intravenous trials. Subjects, with controlled acidic urinary pH, were injected intravenously with (—)-nicotine hydrogen (+)-tartrate (3·07 mg ≡ 1·0 mg base) in sterile aqueous solution (5·0 ml) over 5 min.

Smoking trials. These involved three separate experiments. (i) A subject with controlled acidic urinary pH smoked one cigarette normally. (ii) The same subject, with fluctuating urinary pH, smoked a cigarette normally every half-hour during the course of two working days and urine samples were collected hourly for 36 h. (iii) Subjects with fluctuating urinary pH were allowed to smoke normally and 24 h urine samples were collected starting after the first passage of urine of the morning and continuing until the first passing of urine next morning.

RESULTS AND DISCUSSION

Nicotine-1′-*N*-oxide in urine was reduced quantitatively by titanium trichloride in 5 min at room temperature; a reduction time of up to 24 h did not reduce nicotine or cotinine. A reduction time of 1 h was adopted for the general procedure. Neither constituents in urine nor storage of urine led to interference with the assay procedure. The standard error (s.e. = ±2 standard deviations) of analysis results of replicate samples of nicotine-*N*-oxide in urine was $< \pm 4\%$; that over the calibration range for nicotine-1′-*N*-oxide was ±9·0% and that for nicotine and cotinine, ±5·0%.

Chromatograms of smokers' urine showed two bands corresponding to *cis*- and *trans*-nicotine-1′-*N*-oxide (Booth & Boyland, 1970) (R_F 0·42 and 0·51 respectively)* (Table 1A); more *trans*- than *cis*-diastereoisomer was indicated by visual inspection. Bands corresponding to nicotine (R_F 0·95) and cotinine (R_F 0·82) were also observed. No colour reactions were obtained in the regions where nicotine-di-*N*-oxide or nicotine-aryl-mono-*N*-oxide were located (Table 1A). Elution of bands 6, 7, 8, which span R_F values covering *cis*- and *trans*-nicotine-1′-*N*-oxide, followed by reduction,

Table 1. *R_F values of* (A) *nicotine and some possible metabolites and* (B) *fluorescent bands observed on chromatograms of concentrated smokers urine on Whatman 3MM paper using n-butanol, n-propanol* 2N *ammonium hydroxide* 2:1:1 (v/v) *as developing solvent.*

A		B	
Compound	R_F	Strip No.	R_F of leading edge
Nicotine di-*N*-oxide	0·20	1	0·04
cis-Nicotine-1′-*N*-oxide ..	0·42	2	0·17
trans-Nicotine-1′-*N*-oxide ..	0·51	3	0·22
Nicotine aryl mono-*N*-oxide..	0·77	4	0·30
Cotinine	0·82	5	0·34
Nicotine	0·95	6	0·41
		7	0·42
		8	0·46
		9	0·53
		10	1·0

* *cis*-Nicotine-1′-*N*-oxide denotes *cis*-(methyl/pyridyl).

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gave nicotine (Table 1B). Band 8 (R_F 0·46–0·53) which represented primarily *trans*-nicotine-1′-*N*-oxide gave more nicotine on reduction than did bands 6 and 7 (R_F 0·41–0·46) which represented the R_F value of the *cis*-diastereoisomer with some overlap of the *trans*-band. No other bands contained a substance reduceable to nicotine. Thus more *trans*- than *cis*-nicotine-*N*-oxide is excreted in human urine in agreement with the results of Booth & Boyland (1970).

The urinary excretion of nicotine, after oral administration, was pH dependent, that of cotinine slightly pH-dependent but also volume-dependent, while that of nicotine-1′-*N*-oxide was independent of urinary pH and volume—as expected for a highly polar water-soluble compound (Fig. 1 and Table 2). The rate of urinary

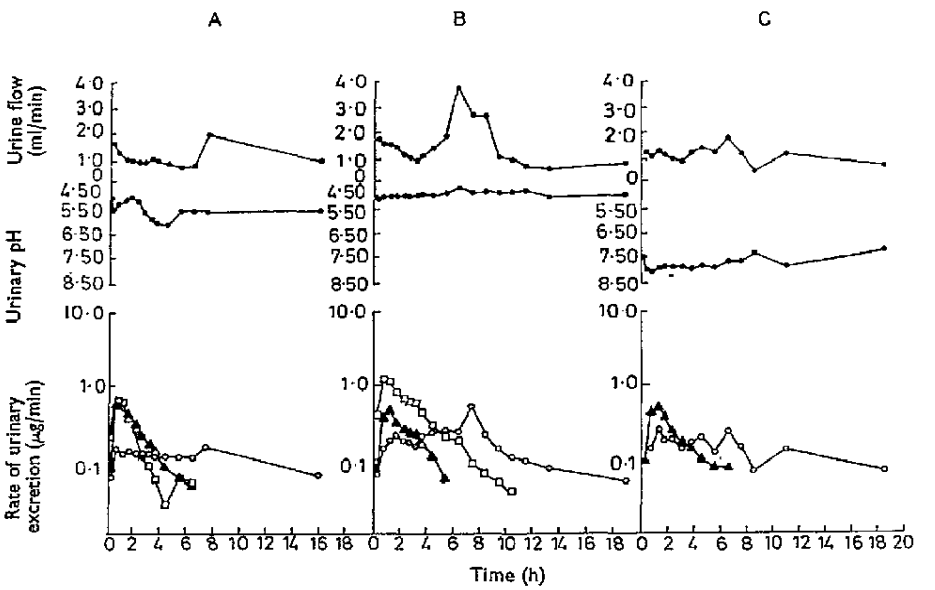

FIG. 1. Urinary excretion of nicotine, cotinine and nicotine-1′-*N*-oxide after oral administration of nicotine (2 mg) to a subject with (A) fluctuating, (B) controlled acidic and (C) controlled alkaline urinary pH. No nicotine excretion under alkaline conditions. □ Nicotine. ▲ Nicotine-1′-*N*-oxide. ○ Cotinine.

Table 2. *The urinary recoveries of nicotine, cotinine and nicotine-1′-N-oxide after oral and intravenous administration of nicotine.*

Route and dose	Subject	Urinary pH	% urinary recovery in 24 h		
			Nicotine	Cotinine	Nicotine-1-*N*-oxide
Oral	1	Uncontrolled	3·8	6·3	4·1
2 mg		Acidic	11·4	9·4	3·6
		Alkaline	0	7·9	4·1
	2	Uncontrolled	0·9	5·1	3·0
		Acidic	11·5	6·3	3·0
		Alkaline	—	—	—
Intravenous	1	Acidic	34·8	21·6	4·2
1 mg	2	Acidic	35·5	20·8	3·8
	3	Acidic	35·4	11·2	3·8

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value was 7 h compared v
excretion, under acidic co
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1A,B), while the cotinine

Table 3. *The urinary rec*
smokers 24 h uri

Subject	Nicot
1	2·5
2	9·0
3	2·7
4	0·2
5	1·9
6	6·3
7	4·7
8	1·5

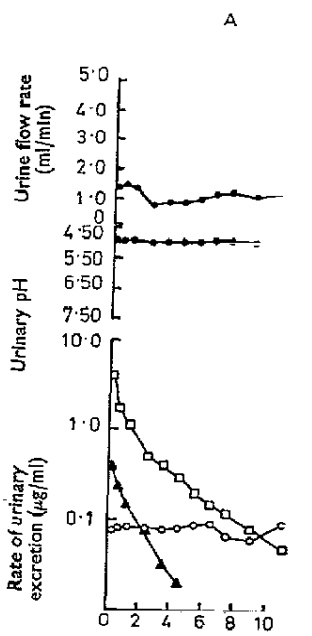

FIG. 2. Urinary excretion of
controlled acidic urinary pH aft
cigarette. □ Nicotine. ▲ Nic

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excretion of cotinine was lower than that of nicotine and of nicotine-1'-*N*-oxide but declined more slowly; the time for the cotinine excretion to fall to half its original value was 7 h compared with 2 h for nicotine and its *N*-oxide. Nicotine-1'-*N*-oxide excretion, under acidic conditions, was complete 6 h after nicotine administration and its peak rate of excretion and excretion profile was parallel to that of nicotine (Fig. 1A,B), while the cotinine excretion profile showed no distinct peak.

Table 3. *The urinary recoveries of nicotine, cotinine and nicotine-1'-N-oxide from smokers 24 h urine samples (fluctuating urinary pH).*

Subject	Recovery from 24 h urine sample (mg)			Ratio
	Nicotine	Cotinine	*N*-oxide	Cotinine/*N*-oxide
1	2·52	5·01	2·46	2·1 : 1
2	9·09	2·76	1·20	2·3 : 1
3	2·72	1·72	0·9	1·9 : 1
4	0·24	0·39	0·11	3·8 : 1
5	1·95	1·46	0·62	2·4 : 1
6	6·32	2·09	2·78	0·75 : 1
7	4·75	2·45	1·03	2·4 : 1
8	1·54	1·74	0·84	2·1 : 1

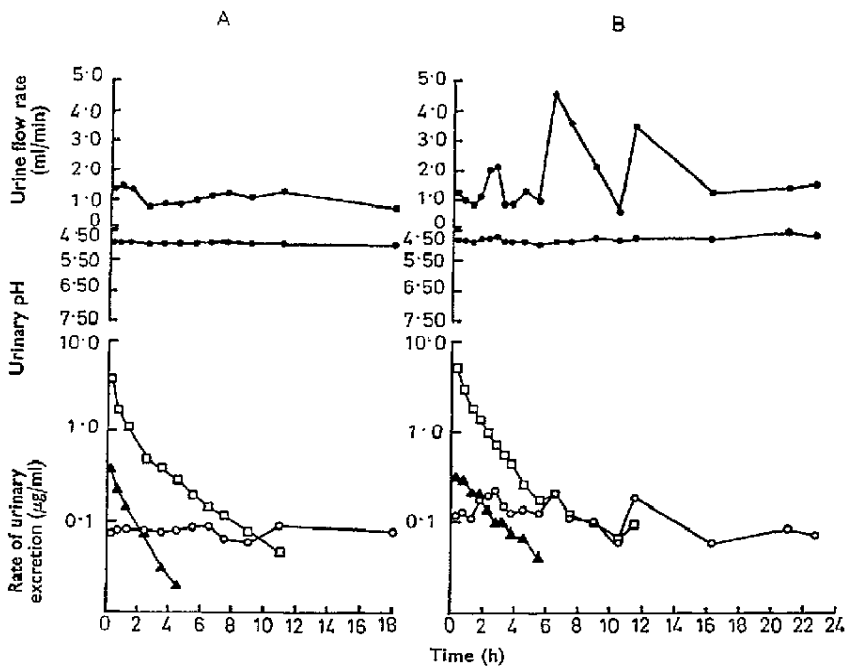

FIG. 2. Urinary excretion of nicotine, cotinine and nicotine-1'-*N*-oxide under conditions of controlled acidic urinary pH after (A) i.v. administration of nicotine (1 mg) and (B) smoking one cigarette. □ Nicotine. ▲ Nicotine-1'-*N*-oxide. ○ Cotinine.

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With uncontrolled urinary pH, the urinary recovery of nicotine was greatly reduced (Fig. 1A) and under alkaline conditions was abolished (Fig. 1C). Urinary cotinine recoveries were altered only slightly by changes of urinary pH and *N*-oxide recoveries were unaffected even though, because of kidney tubular reabsorption, more extensive nicotine metabolism occurred than under acidic urine conditions.

A higher recovery of unchanged nicotine was obtained after intravenous nicotine administration than after oral administration but the recovery of nicotine-1′-*N*-oxide was unaffected (Table 2). Recoveries obtained after intravenous administration of nicotine, however, show inter-subject variation (Beckett, Gorrod & Jenner, 1971). After intravenous administration of nicotine the peak rate of excretion of nicotine and nicotine-1′-*N*-oxide occurred within 15 min; the excretion profiles of nicotine, cotinine and nicotine-1′-*N*-oxide had similar characteristics to those described after oral administration.

Similar urinary recoveries and excretion profiles of nicotine and its metabolites to those observed after intravenous administration have already been shown after rectal nicotine administration (Beckett, Gorrod & Jenner, 1970).

The urinary recovery of nicotine-1′-*N*-oxide, under conditions of acidic urinary pH, from a subject who smoked one cigarette, was small compared to that of nicotine

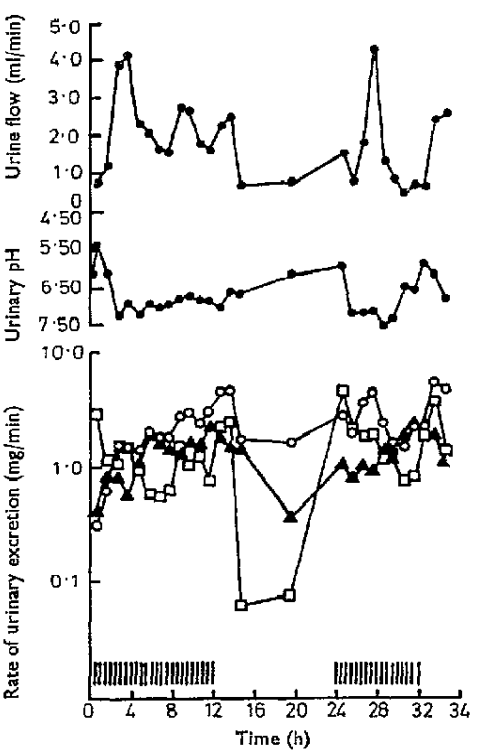

FIG. 3 The urinary excretion of nicotine, cotinine and nicotine-1′-*N*-oxide under conditions of normal fluctuating urinary pH in a subject smoking at regular intervals. □ Nicotine. ▲ Nicotine-1′-*N*-oxide. ○ Cotinine.

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and cotinine (Fig. 2B). However, when one cigarette was smoked every 0·5 h for two working days, under conditions of fluctuating urinary pH, nicotine-1′-*N*-oxide was present in the urine in significant amounts (Fig. 3). Urinary recoveries and excretion profiles of nicotine, nicotine-1′-*N*-oxide and cotinine after intravenous nicotine and cigarette smoke are similar (cf. Fig. 2A and 2B; see also Triggs, 1967).

The amount of nicotine-1′-*N*-oxide excreted by smokers in 24 h under normal conditions was approximately half that of the cotinine excreted (Table 3). Inter-subject variations in the ratio of cotinine to nicotine-1′-*N*-oxide were small. *N*-Oxidation under these conditions, is an important route of metabolism of nicotine.

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